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Published in:
Journal of Bacteriology

DOI:
10.1128/JB.02556-14

Publication date:
2015

JB Journals.ASM.org

The *Escherichia coli* Membrane Protein Insertase YidC Assists in the Biogenesis of Penicillin Binding Proteins

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ABSTRACT

Membrane proteins need to be properly inserted and folded in the membrane in order to perform a range of activities that are essential for the survival of bacteria. The Sec translocon and the YidC insertase are responsible for the insertion of the majority of proteins into the cytoplasmic membrane. YidC can act in combination with the Sec translocon in the insertion and folding of membrane proteins. However, YidC also functions as an insertase independently of the Sec translocon for so-called YidC-only substrates. In addition, YidC can act as a foldase and promote the proper assembly of membrane protein complexes. Here, we investigate the effect of *Escherichia coli* YidC depletion on the assembly of penicillin binding proteins (PBPs), which are involved in cell wall synthesis. YidC depletion does not affect the total amount of the specific cell division PBP3 (FtsI) in the membrane, but the amount of active PBP3, as assessed by substrate binding, is reduced 2-fold. A similar reduction in the amount of active PBP2 was observed, while the levels of active PBP1A/1B and PBP5 were essentially similar. PBP1B and PBP3 disappeared from higher-M_w bands upon YidC depletion, indicating that YidC might play a role in PBP complex formation. Taken together, our results suggest that the foldase activity of YidC can extend to the periplasmic domains of membrane proteins.

IMPORTANCE

This study addresses the role of the membrane protein insertase YidC in the biogenesis of penicillin binding proteins (PBPs). PBPs are proteins containing one transmembrane segment and a large periplasmic or extracellular domain, which are involved in peptidoglycan synthesis. We observe that in the absence of YidC, two critical PBPs are not correctly folded even though the total amount of protein in the membrane is not affected. Our findings extend the function of YidC as a foldase for membrane protein (complexes) to periplasmic domains of membrane proteins.

Membrane proteins need to be properly inserted and folded in the membrane in order to be functional. The *Escherichia coli* Sec translocon and the YidC insertase are involved in the insertion of the majority of membrane proteins into the membrane. YidC can act in combination with the Sec translocon to facilitate the insertion and folding of membrane proteins, but can also function on its own as an insertase for so-called YidC-only substrates (1). Although YidC was discovered more than 13 years ago (2, 3), only a few YidC-only substrates are known at present. YidC-only substrates have short translocated regions and include the F_1F_0-ATPase subunit c (4–7), the M13 phage procoat protein (8), the mechano-sensing MscL protein (9), the Sci-1 type VI secretion system subunit TssL (10), and the Pf3 coat protein (3).

In addition to its role as an insertase, YidC can also act as a foldase for some proteins such as the sugar transporter LacY (11, 12) and mediate the proper assembly of membrane protein complexes such as the $MalFGK_2$ maltose transporter (13) and the MscL homopentameric pore (14). This feature might be related to the capacity of YidC to interact with the transmembrane domains of proteins that are released by the Sec translocon, whereupon YidC would facilitate the correct assembly and interaction of the transmembrane helices (15, 16). A major step in understanding the mode of action of YidC was accomplished with the elucidation of its crystal structure, providing insights on how single-spanning proteins may be inserted in the membrane involving a positively charged hydrophilic groove of YidC (17).

To identify novel YidC substrates and to assess the role of YidC in the assembly of membrane protein complexes, inner membrane proteomes of *E. coli* cells with or without YidC have been analyzed by various groups. Depletion of YidC results in an increased chaperone response, a reduction in the levels of protein complexes, and a lower level of membrane proteins with polar domains smaller than 100 amino acids (18, 19). In addition, YidC depletion results in changes in overall gene expression and increased cell length (20). The latter, also known as filamentation, is often associated with defects in cell division or cell wall synthesis. Most proteins involved in cell division and cell wall synthesis are present in small amounts and are generally not identified in membrane proteomic studies, and yet many contain at least one transmembrane segment and are thought to be part of large protein complexes (21). Nevertheless, an earlier study on YidC-depleted cells revealed a decrease of the cell division associated ATP-binding cassette transporter-like proteins FtsEX that activates cell wall

Received 8 December 2014 **Accepted** 2 February 2015

Accepted manuscript posted online 9 February 2015

Citation de Sousa Borges A, de Keyzer J, Driessen AJM, Scheffers D-J. 2015. The *Escherichia coli* membrane protein insertase YidC assists in the biogenesis of penicillin binding proteins. J Bacteriol 197:1444–1450. doi:10.1128/JB.02556-14.

Editor: P. de Boer

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.02556-14.

doi:10.1128/JB.02556-14

hydrolysis during division (18, 22). In contrast, the levels of penicillin binding protein 5 (PBP5) and PBP6 increased upon YidC depletion (18). The cell division protein FtsQ was not identified as a YidC substrate in proteomic studies but is often used as a substrate for *in vitro* protein translocation studies (18, 23). *In vitro*, the activities of SecYEG, the SecA translocation ATPase, and the proton motive force are sufficient for the correct and complete membrane insertion of FtsQ (24). However, a kinetic effect of YidC cannot be excluded since YidC was found to interact with the transmembrane segment of FtsQ when the FtsQ nascent chain bound to ribosomes was used as a substrate in *in vitro* insertion experiments (25, 26).

In the present study, we focused on PBPs involved in cell division and cell wall synthesis. PBPs play a crucial role in cell growth by adding new peptidoglycan (PG) to the cell wall and in cell division by synthesizing new PG between two daughter cells. PBPs can be divided into two main groups, the high-molecular-weight (high-M_w) and the low-M_w PBPs. *E. coli* high-M_w PBPs are subdivided into class A PBPs (1A/1B/1C) that have both transglycosylase and transpeptidase activity and class B PBPs (2/3) that only have transpeptidase activity. Class B PBPs define the directionality of peptidoglycan (PG) growth, with PBP2 being required for cell elongation and PBP3 (or FtsI) required for cell division (27). Low-M_w PBPs include type 4 (PBP4) and type 5 (PBP5/6/6b) carboxypeptidases, that are involved in PG modification, recycling, and separation (28). Several high-M_w PBPs have been found to interact with either themselves or other PBPs, forming complexes involved in cell division or cell elongation (21). PBP1B is known to dimerize (29) and to interact with PBP3 (30). PBP3 also forms dimers (31, 32), and interacts with PBP2 (33). PBPs can collectively be labeled and visualized using Bocillin-FL, a fluorescent derivative of penicillin that covalently binds to their active sites (34). Importantly, binding of Bocillin-FL is also an indication for correct folding of the active site present in the (trans)peptidase domain of a PBP (34). It has to be noted that not all PBPs are detected by Bocillin-FL, since the affinity for Bocillin-FL varies per PBP and per organism. In *E. coli*, PBP1A/B, PBP2, PBP3, PBP4, and PBP5 are readily labeled and detected. Therefore, these PBPs were the focus of our study.

We show here that YidC depletion does not affect the total level of PBP3 in the membrane but that the amount of correctly folded PBP3 is reduced. A similar reduction in the level of folded PBP2 was observed, while the levels of other PBPs remained essentially unaltered. Our results suggest that the foldase activity of YidC can extend to the periplasmic domains of membrane proteins.

MATERIALS AND METHODS

Strains and media. *E. coli* strains FTL10 [MC4100-A; Δ*yidC attB*::(*araC* P_{BAD} *yidC*$^+$) Kan^r] (35) and JOE417 (MC4100; *araD*$^+$ *ftsQE14*::*kan*/pBAD33-*ftsQ*) (36) were used for depletion experiments. *E. coli* DH5α was used for cloning. Strains were grown at 37°C on solid medium (LB broth Lennox plus agar) and liquid medium (LB broth Lennox), with 1% NaCl when required. When necessary, ampicillin (100 μg/ml), kanamycin (25 μg/ml), or chloramphenicol (25 μg/ml) were added. When appropriate, lysogeny broth Lennox (LB) medium was supplemented with 0.2% (wt/vol) of glucose or arabinose.

Cloning. The coding sequence for *ftsI* was amplified from *E. coli* DH5α chromosomal DNA using the primer pair (5′-CGGCGGCATATG AAAGCAGCGGCGAAAAC-3′ and 5′-GCCGCCGGATCCTTACGATC TGCCACCTGTC-3′) and cloned into the pGEM-T vector (Promega). An internal NdeI restriction site was removed from *ftsI* by QuickSite site-directed mutagenesis with the primer pair (5′-GCACCGTGGTGCACAT GATGGAAAGCG-3′ and 5′-CGCTTTCCATCATGTGCACCACGGTG C-3′) according to the manufacturer's protocol (Stratagene), resulting in a silent replacement of thymine 1406 for a cytosine (*ftsI**). After NdeI/BamHI digestion, *ftsI** was cloned into NdeI/BamHI digested pET-20b(+) (Novagen), resulting in plasmid pDJ125. The final construct was confirmed by sequencing.

YidC and FtsQ depletion. To generate cells depleted of YidC or FtsQ, the *E. coli* bacterial strains FTL10 (35) and JOE417 (36) were used, in which the expression of either *yidC* or *ftsQ* is under the control of the arabinose promoter. YidC and FtsQ depletions were carried out as previously described (18, 37) with minor alterations. A single colony of FTL10 or JOE417 was precultured in 5 ml of LB medium with 0.2% arabinose and 25 μg of kanamycin/ml, plus 25 μg of chloramphenicol/ml for JOE417, at 37°C during the day. Preculture was diluted 100-fold with fresh LB medium containing the same supplements, and growth was continued overnight at 37°C. The overnight culture was then diluted 100 times into warm LB medium with 0.2% arabinose and grown at 37°C until an A_{600} of 0.6 to 0.8 was reached. The cells were washed in warm LB medium and concentrated 3.3-fold with warm LB medium. For control cells, the concentrated suspension was diluted 200-fold in warm LB medium containing 0.2% arabinose, grown to an A_{600} of 0.8, and collected. For depleted cells, the concentrated suspension was diluted 25-fold in warm LB medium containing 0.2% glucose (depleted cells) and grown until reaching an A_{600} of 0.6 to 0.8, at which point the depleted cells were diluted two times with warm LB containing 0.2% of glucose so that the culture remained in the exponential phase. This process was repeated until the depleted cells ceased to grow, at which moment the cells were collected. After collection (8,950 × *g*, 15 min, 4°C), the cells were resuspended in 50 mM Tris-HCl (pH 8.0) with 20% sucrose, flash frozen in liquid nitrogen, and stored at −80°C. Three independent depletions were performed with each strain.

IMV isolation. Inner membrane vesicles (IMVs) were obtained as previously described (38), with some alterations. Frozen cells were thawed and passed through a cell disruptor (Constant Systems One Shot; LA Biosystems) twice at 13,000 lb/in^2. Centrifugation was used to remove unbroken cells (2,095 × *g*, 10 min, 4°C) and debris (7,649 × *g*, 5 min, 4°C). Membranes were collected from the supernatant at 186,010 × *g* for 60 min. at 4°C and resuspended in 50 mM Tris-HCl (pH 8.0). The membrane suspension was loaded on top of a sucrose gradient composed of 1 ml of 55% (wt/vol) sucrose, 1.8 ml of 51% (wt/vol) sucrose, 0.8 ml of 45% (wt/vol) sucrose, and 0.8 ml of 36% (wt/vol) sucrose in 50 mM Tris-HCl (pH 8.0). After centrifugation (444,000 × *g*, 30 min, 4°C), the IMVs, visible as a brown band in the 45% sucrose fraction of the gradient, were collected and diluted into 50 mM Tris-HCl (pH 8.0). The IMVs were harvested after centrifugation (186,010 × *g*, 60 min, 4°C) and resuspended in 50 mM Tris-HCl (pH 8.0) with 20% glycerol. Purified IMVs were flash frozen and stored at −80°C. Total protein concentration was determined using the Bio-Rad DC protein assay kit with bovine serum albumin as the standard.

Bocillin labeling. IMVs were equalized for protein concentration and incubated with 7.5 μM Bocillin-FL (Life Technologies) for 15 min at 23°C with shaking. To stop the labeling reaction, SDS-PAGE loading buffer was added, and samples were incubated for 5 min at 95°C. Samples were analyzed by using SDS–10% PAGE. Fluorescent bands were visualized using a Typhoon Trio (GE Healthcare) scanner with 488 and 526 nm as the excitation and emission wavelengths, respectively. A prestained protein ladder (Thermo Scientific) was visualized using 532 and 670 nm as the excitation and emission wavelengths, respectively. The experiment was performed for each set (three in total) of depleted IMVs. Fluorescent bands were quantified by using ImageQuant TL 2003.v3 (Amersham Biosciences).

Immunoblotting. Equal amounts of protein were loaded and separated using SDS–10% PAGE and transferred to a polyvinylidene difluoride membrane according to standard protocols. The primary antibodies

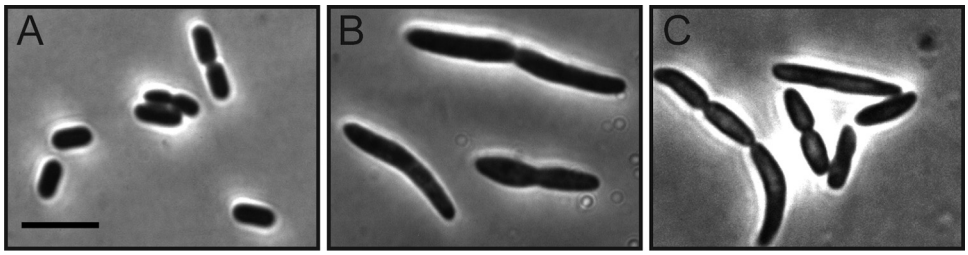


FIG 1 Cell morphology is altered upon YidC depletion. (A to C) Bright-field microscopy images of fixed FTL10 cells after growth in LB medium with 0.2% arabinose to express *yidC* (A), fixed FTL10 cells after growth in LB medium with 0.2% glucose to repress the expression of *yidC* (B), and fixed FTL10 cells after growth in LB medium with 1% NaCl and 0.2% glucose (C). Scale bar, 5 μm.

for protein detection were anti-YidC, anti-FtsZ (lab collection), anti-PBP3, anti-FtsQ, anti-LepB (a gift from J. W. de Gier), anti-PBP1a, anti-PBP1B (a gift from W. Vollmer), and anti-PBP5 (a gift from T. den Blaauwen). Alkaline-phosphatase-conjugated secondary antibodies were obtained from Sigma-Aldrich. Blots were developed using the chemiluminescent substrate CDP-Star (Roche), and chemiluminescence was detected by using a Fujifilm LAS 4000 imager (GE Healthcare). Images were analyzed and band intensities were quantified using the software AIDA 4.22.034 (Raytest).

Data analysis. Each depletion experiment was performed three times, using independent cultures. IMVs derived from the YidC depletion experiment were analyzed twice in independent bocillin labeling and immunoblotting experiments, resulting in a total of six intensity values per PBP, for both bocillin labeling and immunodetection. A Kolmogorov-Smirnov test was applied to determine whether the distribution of the data is significantly different from the normal distribution. Subsequently, a paired *t* test was used to determine significant differences in the bocillin signal intensities between YidC-depleted and -nondepleted IMVs, and a Wilcoxon matched pairs test was used to determine significant differences in the immunoblotting signal intensities between YidC-depleted and -nondepleted IMVs. The signal intensities between the FtsQ-depleted and -nondepleted IMVs were found to be not significantly different ($P > 0.05$) using a Wilcoxon matched-pairs test. Changes in protein level are depicted as the ratio of band intensity in nondepleted IMVs over band intensity in depleted IMVs. Statistical analysis was done using SPSS software (IBM SPSS Statistics, version 22) and Microsoft Excel.

***In vitro* PBP3 insertion.** PBP3 was expressed from plasmid pDJ125 and synthesized as described previously (39) using a S135 lysate prepared from the RNase I-deficient strain *E. coli* A19 (40), T7 polymerase (Fermentas), and an Easytag express ^{35}S protein labeling mix (Perkin-Elmer Life Sciences). When indicated, reactions were supplemented with 50 μg of IMVs/ml. After 30 min, a 10% synthesis control was collected, and the remainder of the reaction mixture was treated with 6 M urea (final concentration) for 30 min on ice to assay membrane integration (41). Vesicles were sedimented by ultracentrifugation at 150,000 × *g* for 30 min, after which the supernatant was removed, and the pellet was resuspended in SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE, and ^{35}S-labeled proteins were detected by phosphorimaging. The experiment was performed twice using independent IMV preparations.

Microscopy. Samples for microscopy analysis were collected during YidC depletion. Samples containing 1 ml of culture were taken from the control and YidC-depleted cells and fixed with 1 ml of 8% formaldehyde for 30 min at room temperature. After incubation, cells were washed two times in phosphate-buffered saline (PBS; 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl [pH 7.3]) and resuspended in a final volume of 100 μl of PBS. Fixed cells were mounted on an agarose pad (1% [wt/vol] in PBS) and visualized using a Nikon Ti-E microscope (Nikon Instruments, Tokyo, Japan) equipped with a Hamamatsu Orca Flash4.0 camera. Image analysis was performed using the software ImageJ (http://rsb.info.nih.gov/ij/).

RESULTS AND DISCUSSION

YidC depletion influences cell morphology. The effects of YidC depletion on cell morphology were analyzed using bright-field microscopy. YidC depletion resulted in cells that were both longer and wider compared to the control (Fig. 1A and B), as previously described for a different YidC depletion strain (20). This aberrant shape is generally associated with defects in cell wall synthesis and impaired cell division. YidC depletion affects the levels of the ABC transporter FtsEX that is involved in cell wall hydrolysis during division (18, 22). To test whether the observed phenotype can be attributed to FtsEX, YidC depletion was also carried out in medium containing 1% NaCl since a high salt concentration recovers the elongated cell phenotype of *ftsEX* knockout cells (42). Since cells depleted in the presence of 1% NaCl remained both longer and wider, it is unlikely that the phenotype is caused by reduced levels of FtsEX (Fig. 1C). Therefore, we decided to analyze the effect of YidC depletion on the assembly of PBPs in the membrane.

Class B PBPs require YidC for proper folding. PBPs are a group of inner membrane proteins involved in cell wall synthesis during growth and cell division. In order to investigate the effect of YidC depletion on the PBPs, we labeled IMVs with or without YidC with the fluorescent penicillin analogue Bocillin-FL and detected the patterns of fluorescently labeled PBPs by SDS-PAGE (Fig. 2A). Efficient YidC depletion was assessed by immunoblotting (Fig. 2B), and equal amounts of loaded protein were confirmed by comparison of the levels of the inner membrane protein leader peptidase (LepB) (Fig. 2B and Table 1), which remained constant upon YidC depletion (43). The absence of YidC resulted in the disappearance of some fluorescent bands running at a molecular mass higher than 100 kDa, which is indicative of complexes of PBPs (with themselves or other proteins) that have not been completely disintegrated upon treatment with heat and sample buffer (Fig. 2A). Quantification of the fluorescence associated with the bands revealed that Bocillin-FL labeled class B PBPs 2 and 3 were >2-fold reduced in the absence of YidC and that the difference between the samples from nondepleted and depleted cells was significant (Table 1). Also, PBP4 was nearly 2-fold reduced. Although the reduction was robust in the gels, this difference was not significant. The levels of PBP1 and PBP5 were essentially not affected by YidC depletion (Table 1).

The decrease in the levels of labeled PBP2 and PBP3 can be caused by either a decrease of the total amounts of these proteins in the membrane or by a reduced folding of the protein, resulting in a misfolded transpeptidase domain that no longer binds Bocil-

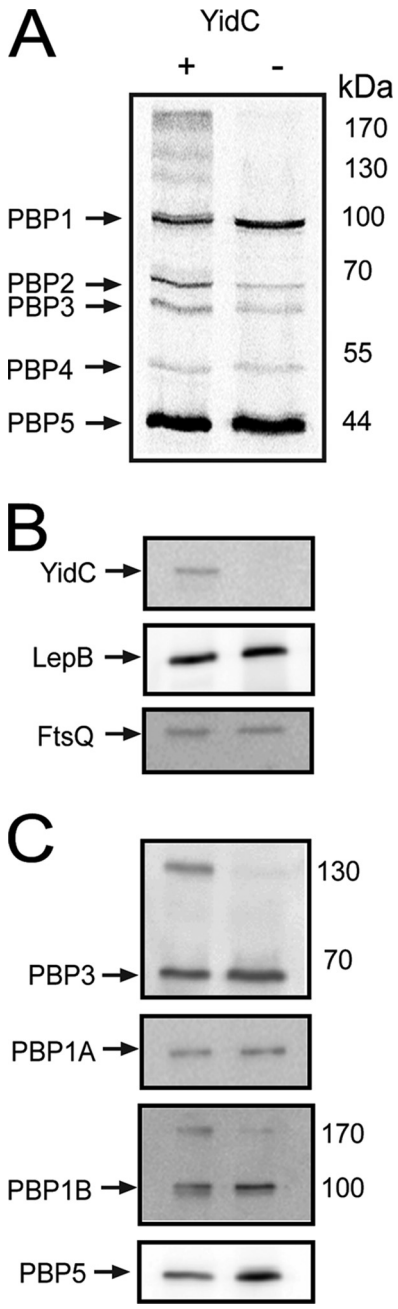


FIG 2 Bocillin-FL labeling and immunoblot analysis of IMVs with or without YidC. (A) SDS-PAGE showing PBP profiles of Bocillin-FL-labeled IMVs. Molecular masses are indicated on the right. (B and C) Immunoblot analysis of IMVs with or without YidC with antibodies directed against YidC, LepB, FtsQ, PBP3, PBP1A, PBP1B, and PBP5. Three independent IMV preparations essentially gave the same results (quantification in Table 1).

lin-FL. Therefore, we assessed the levels of various PBPs in the IMVs by immunoblotting. The levels of PBP1A, PBP1B, and PBP3 were essentially similar (Fig. 1C and Table 1). PBP5 levels appeared to be slightly increased upon YidC depletion (Fig. 1C), in agreement with an early study (18); however, this difference was not significant (Table 1). Both PBP3 and PBP1B antibodies cross-reacted with two bands, one corresponding to a PBP3 or PBP1B monomer and one at a higher M_w that was reduced upon YidC depletion. The absence of specific antibodies prevented us from performing the similar experiments for PBP2 and PBP4.

PBP3 and PBP1B are known to interact (30). The presence of both PBP3 and PBP1B in higher-M_w complexes and the disappearance of these bands upon YidC depletion suggested that YidC might facilitate this interaction. Careful analysis of the high-M_w bands found with antibodies against PBP1B and PBP3 revealed that these bands do not overlap each other (although they overlap the bocillin-labeled high-M_w bands), suggesting that they do not correspond to a putative PBP3/PBP1B complex (see Fig. S1 in the supplemental material). The absence of the PBP3/PBP1B complex is probably caused by complex disruption during sample preparation as the nondepleted sample also did not show overlapping bands of PBP3 and PBP1B (see Fig. S1 in the supplemental material).

The high-M_w bands may correspond to PBP1B and PBP3 homodimers or to complexes of PBP1B and PBP3 involving other, as-yet-unknown proteins. PBP3 was shown to homodimerize *in vivo* (32) and the crystal structure of the periplasmic domain of PBP3 was recently determined, supporting the evidence of PBP3 dimers (31). PBP1B, a bifunctional transglycosylase enzyme, is known to form dimers *in vivo* (44) that are able to synthesize glycan chains and cross-link the peptide bridges *in vitro* (29). As outlined above, we note that our findings do not exclude the possibility that the high-M_w bands correspond to complexes containing PBP1B and PBP3 with other proteins. However, given that both proteins form dimers *in vivo* we think that the high-M_w bands containing PBP3 and PBP1B correspond to homodimers and that YidC is involved in formation or stabilization of PBP dimers.

Together, these results indicate that in the absence of YidC the total levels of PBPs in the membrane are hardly affected but that at least a considerable fraction of the class B PBPs PBP2 and PBP3 no longer bind a fluorescent substrate analog, an observation indicative of misfolding of the periplasmic peptidase domain. The absence of high-M_w forms of PBP1B and PBP3 suggests that their capacity for complex formation is disturbed. This suggests that the

TABLE 1 Ratios of bocillin-labeled or immunodetected proteins present in IMVs upon YidC or FtsQ depletion[a]

Protein	Avg ratio (nondepeleted/depleted IMVs) ± SEM[b]			
	YidC$^+$/YidC$^-$		FtsQ$^+$/FtsQ$^-$	
	Bocillin labeling	Immunoblotting	Bocillin labeling	Immunoblotting
YidC	ND	ND	ND	1.02 ± 0.02
FtsQ	ND	1.16 ± 0.18	ND	ND
LepB	ND	0.97 ± 0.03	ND	1.1 ± 0.1
PBP1A	1.13 ± 0.18	1.19 ± 0.18	0.94 ± 0.09	0.99 ± 0.17
PBP1B	1.13 ± 0.18	1.07 ± 0.09	0.94 ± 0.09	0.81 ± 0.13
PBP2	2.43 ± 0.21*	ND	0.89 ± 0.14	ND
PBP3	2.06 ± 0.35*	1.17 ± 0.11	1.17 ± 0.19	1.2 ± 0.11
PBP4	1.86 ± 0.34	ND	1.12 ± 0.09	ND
PBP5	1.15 ± 0.23	0.88 ± 0.07	1.36 ± 0.34	1.00 ± 0.17

[a] YidC$^+$/YidC$^-$ IMVs (three independent preparations, in duplicate) and FtsQ$^+$/FtsQ$^-$ IMVs (three independent preparations) were tested, and the band intensities were quantified. The ratio of proteins in nondepleted IMVs to proteins in depleted IMVs was determined. The resulting average ratios and standard errors of the mean are shown.
[b] *, samples in which the levels of detected protein were significantly different; ND, not determined.

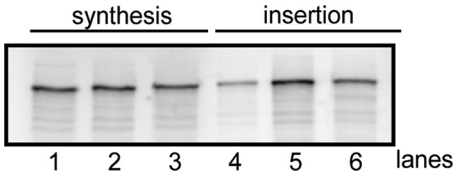


FIG 3 PBP3 membrane insertion is independent of YidC. *In vitro* membrane insertion assay using YidC-depleted and control IMVs. ^{35}S-labeled PBP3 was synthesized in the absence of IMVs (lanes 1 and 4) or in the presence of IMVs with (lanes 3 and 6) or without YidC (lanes 2 and 5). *In vitro* synthesis of ^{35}S-labeled PBP3 was similar irrespective of the presence of IMVs (lane 1 to 3, 10% of the total synthesis for each reaction). Subsequently, IMVs were incubated with 6 M urea, followed by recovery of the IMVs by ultracentrifugation. The presence of membrane inserted ^{35}S-labeled PBP3 in the pellet fractions was analyzed (lanes 4 to 6). The experiment was performed twice using independent IMV preparations, and a representative result is shown.

YidC foldase activity (11–14) can extend to the periplasmic domains of membrane-anchored proteins.

PBP3 insertion is not dependent on YidC. The presence of PBP3 in YidC depleted IMVs (Fig. 2C) suggested that, similar to other monotopic membrane proteins with periplasmic domains (24), PBP3 does not require YidC for membrane insertion. We performed an *in vitro* synthesis and membrane insertion assay for PBP3 using YidC-depleted IMVs. *In vitro*-synthesized PBP3 inserted into the membrane of IMVs independent of the presence of YidC (Fig. 3). This experiment confirms that YidC is not required for membrane insertion of PBP3.

PBP2 and PBP3 folding does not depend on FtsQ. PBP3 is an essential cell division protein that requires FtsQ for localization to the cell division site (45). Since FtsQ has been reported to interact with YidC (24–26), we considered the possibility that the effect on PBP3 folding upon YidC depletion is indirect, resulting from improper assembly of FtsQ. To exclude this possibility, we first determined the levels of FtsQ upon YidC depletion and observed similar levels of FtsQ in YidC-depleted cells (Fig. 2B and Table 1). This suggests that FtsQ does not require YidC for its assembly, a finding in agreement with previous findings that showed that membrane insertion of FtsQ is not affected in the absence of YidC (23) and that FtsQ requires only SecYEG and the proton motive force for proper insertion (24). Second, the effects of FtsQ depletion on PBPs were investigated by Bocillin-FL labeling and immunoblotting of IMVs isolated from cells depleted for FtsQ. Controls showed depletion of FtsQ, with similar levels for LepB and YidC, indicating equal loading (Fig. 4B and Table 1). Bocillin-FL labeling of IMVs of FtsQ-depleted cells did not show substantial differences in the levels of labeled PBPs. Fluorescent bands above 100 kDa were not absent but appeared to be reduced upon FtsQ depletion. Immunoblotting confirmed that the levels of PBP1A and PBP5 were similar in these samples (Fig. 4C and Table 1). The slight disappearance of the high-M_w bands, corresponding to complexes, in the absence of FtsQ could be due to a reduction of FtsQ-PBP3 complexes or other membrane protein complexes involved in cell division (46). Our results show that the folding defects in PBP3 (and other PBPs) upon YidC depletion are not an indirect effect of an FtsQ assembly defect.

Conclusion. Here, we report that the correct folding of the class B transpeptidases PBP2 and PBP3 is dependent on the presence of the membrane protein insertase/foldase YidC. Depletion of YidC leads to wider cells that have a division defect, which is in

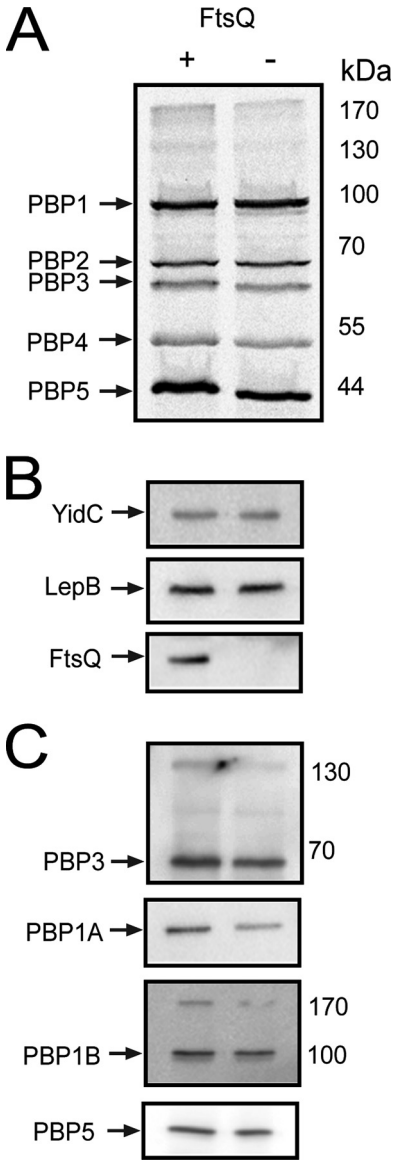


FIG 4 Bocillin-FL labeling and immunoblot analysis of IMVs with or without FtsQ. (A) SDS-PAGE showing PBP profiles of Bocillin-FL labeled IMVs. Molecular masses are indicated on the right. (B and C) Immunoblot analysis of IMVs with or without FtsQ with antibodies directed against YidC, LepB, FtsQ, PBP3, PBP1A, PBP1B, and PBP5. Three independent IMV preparations essentially gave the same results (quantification in Table 1).

accordance with a reduction in function of the elongation and division specific class B transpeptidases PBP2 and PBP3. Importantly, whereas earlier reports already identified YidC as a foldase/chaperone for proteins with multiple transmembrane segments (11–14), we report here a role for YidC in the correct assembly of periplasmic domains of membrane proteins. We observed that the absence of YidC hardly affects the total amount of PBP3 in the membrane but more than halves the amount of correctly folded PBP3, as determined by substrate binding. The amount of correctly folded PBP5 is also slightly decreased even though the total amount of PBP5 increases upon YidC depletion, as noted earlier (18). Transcriptome analysis showed a 2.45-fold upregulation of *dacA* (which codes for PBP5) transcription upon YidC depletion

(20), but it is not clear whether this is a direct effect of the diminished amount of correctly folded PBP5 in the cells or caused by another regulatory mechanism. PBP6 levels have also been found to increase upon YidC depletion (18), but since PBP6 is not labeled with Bocillin-FL, we have not been able to determine whether YidC depletion affects PBP6 folding.

We also note that various PBPs are no longer present in higher-M_w complexes in the absence of YidC. *Bacillus subtilis* class B PBPs also require a chaperone, the lipoprotein PrsA (47) for correct folding. PrsA is a peptidyl-prolyl *cis-trans* isomerase that is conserved in Gram-positive organisms. It may be that the effect of YidC on PBP folding in *E. coli* is indirect, with YidC required for folding of a factor that aids in PBP biogenesis. Nevertheless, such a factor would also (predominantly) be located in the periplasm, and thus this would still extend the role of YidC to the assembly of periplasmic protein domains. It remains to be determined how exactly YidC exerts its function as a foldase.

ACKNOWLEDGMENTS

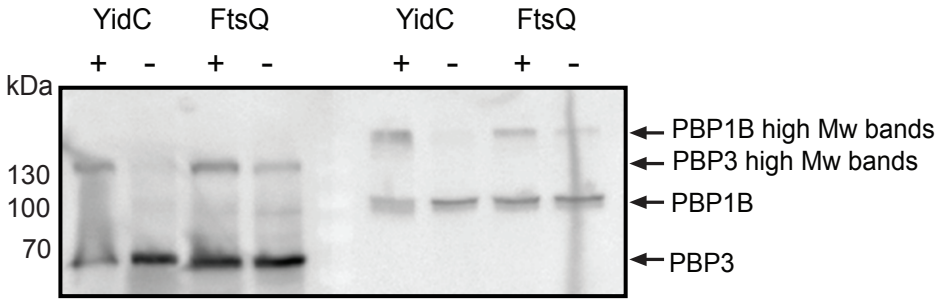
We are grateful to Waldemar Vollmer (The Centre for Bacterial Cell Biology, Newcastle University, Newcastle, United Kingdom), Jan-Willem de Gier (Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden), Tanneke den Blaauwen (Department of Bacterial Cell Biology, University of Amsterdam, Amsterdam, The Netherlands), and Mohammed Terrak (Center for Protein Engineering, University of Liège, Liège, Belgium) for the generous gift of antibodies. We thank Joana Falcão Salles (Department of Microbial Ecology, University of Groningen, Groningen, The Netherlands) for help with the statistical analysis.

This study was funded by a doctoral grant to A.D.S.B. (SFRH/BD/78061/2011) from POPH/FSE and FCT (Fundação para a Ciência e Tecnologia) from Portugal and by a VIDI grant (D.-J.S.) from The Netherlands Organization for Scientific Research (NWO).

REFERENCES

1. **Du Plessis DJ, Nouwen N, Driessen AJ.** 2011. The s translocase. Biochim Biophys Acta **1808:**851–865. http://dx.doi.org/10.1016/j.bbamem.2010.08.016.
2. **Scotti PA, Urbanus ML, Brunner J, de Gier JL, von Heijne G, van der Does C, Driessen AJ, Oudega B, Luirink J.** 2000. YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. EMBO J **19:**542–549. http://dx.doi.org/10.1093/emboj/19.4.542.
3. **Samuelson JC, Chen M, Jiang F, Möller I, Wiedmann M, Kuhn A, Phillips GJ, Dalbey RE.** 2000. YidC mediates membrane protein insertion in bacteria. Nature **406:**637–641. http://dx.doi.org/10.1038/35020586.
4. **van Bloois E, Jan Haan G, J de Gier Oudega B, Luirink J.** 2004. F_1 F_0 ATP synthase subunit c is targeted by the SRP to YidC in the *Escherichia coli* inner membrane. FEBS Lett **576:**97–100. http://dx.doi.org/10.1016/j.febslet.2004.08.069.
5. **Yi L, Celebi N, Chen M, Dalbey RE.** 2004. Sec/SRP requirements and energetics of membrane insertion of subunits a, b, and c of the *Escherichia coli* F_1F_0 ATP synthase. J Biol Chem **279:**39260–39267. http://dx.doi.org/10.1074/jbc.M405490200.
6. **van der Laan M, Bechtluft P, Kol S, Nouwen N, Driessen AJ.** 2004. F_1F_0 ATP synthase subunit c is a substrate of the novel YidC pathway for membrane protein biogenesis. J Cell Biol **165:**213–222. http://dx.doi.org/10.1083/jcb.200402100.
7. **Yi L, Jiang F, Chen M, Cain B, Bolhuis A, Dalbey RE.** 2003. YidC is strictly required for membrane insertion of subunits a and c of the F_1F_0ATP synthase and SecE of the SecYEG translocase. Biochemistry **42:**10537–10544. http://dx.doi.org/10.1021/bi034309h.
8. **Samuelson JC, Jiang F, Yi L, Chen M, J de Gier Kuhn A, Dalbey RE.** 2001. Function of YidC for the insertion of M13 procoat protein in *Escherichia coli*: translocation of mutants that show differences in their membrane potential dependence and Sec requirement. J Biol Chem **276:**34847–34852. http://dx.doi.org/10.1074/jbc.M105793200.
9. **Facey SJ, Neugebauer SA, Krauss S, Kuhn A.** 2007. The mechanosensitive channel protein MscL is targeted by the SRP to the novel YidC membrane insertion pathway of *Escherichia coli*. J Mol Biol **365:**995–1004. http://dx.doi.org/10.1016/j.jmb.2006.10.083.
10. **Aschtgen M, Zoued A, Lloubès R, Journet L, Cascales E.** 2012. The C-tail anchored TssL subunit, an essential protein of the enteroaggregative *Escherichia coli* Sci-1 type VI secretion system, is inserted by YidC. MicrobiologyOpen **1:**71–82. http://dx.doi.org/10.1002/mbo3.9.
11. **Zhu L, Kaback HR, Dalbey RE.** 2013. YidC protein, a molecular chaperone for LacY protein folding via the SecYEG protein machinery. J Biol Chem **288:**28180–28194. http://dx.doi.org/10.1074/jbc.M113.491613.
12. **Nagamori S, Smirnova IN, Kaback HR.** 2004. Role of YidC in folding of polytopic membrane proteins. J Cell Biol **165:**53–62. http://dx.doi.org/10.1083/jcb.200402067.
13. **Wagner S, Pop OI, Haan GJ, Baars L, Koningstein G, Klepsch MM, Genevaux P, Luirink J, de Gier JW.** 2008. Biogenesis of MalF and the $MalFGK_2$ maltose transport complex in *Escherichia coli* requires YidC. J Biol Chem **283:**17881–17890. http://dx.doi.org/10.1074/jbc.M801481200.
14. **Pop OI, Soprova Z, Koningstein G, Scheffers D, van Ulsen P, Wickström D, de Gier JW, Luirink J.** 2009. YidC is required for the assembly of the MscL homopentameric pore. FEBS J **276:**4891–4899. http://dx.doi.org/10.1111/j.1742-4658.2009.07188.x.
15. **Beck K, Eisner G, Trescher D, Dalbey RE, Brunner J, Müller M.** 2001. YidC, an assembly site for polytopic *Escherichia coli* membrane proteins located in immediate proximity to the SecYE translocon and lipids. EMBO Rep **2:**709–714. http://dx.doi.org/10.1093/embo-reports/kve154.
16. **Dalbey RE, Kuhn A.** 2004. YidC family members are involved in the membrane insertion, lateral integration, folding, and assembly of membrane proteins. J Cell Biol **166:**769–774. http://dx.doi.org/10.1083/jcb.200405161.
17. **Kumazaki K, Chiba S, Takemoto M, Furukawa A, Nishiyama K, Sugano Y, Mori T, Dohmae N, Hirata K, Nakada-Nakura Y.** 2014. Structural basis of Sec-independent membrane protein insertion by YidC. Nature **509:**516–520. http://dx.doi.org/10.1038/nature13167.
18. **Price CE, Otto A, Fusetti F, Becher D, Hecker M, Driessen AJ.** 2010. Differential effect of YidC depletion on the membrane proteome of *Escherichia coli* under aerobic and anaerobic growth conditions. Proteomics **10:**3235–3247. http://dx.doi.org/10.1002/pmic.201000284.
19. **Wickström D, Wagner S, Simonsson P, Pop O, Baars L, Ytterberg AJ, van Wijk KJ, Luirink J, de Gier JW.** 2011. Characterization of the consequences of YidC depletion on the inner membrane proteome of *Escherichia coli* using 2D blue native/SDS-PAGE. J Mol Biol **409:**124–135. http://dx.doi.org/10.1016/j.jmb.2011.03.068.
20. **Wang P, Kuhn A, Dalbey RE.** 2010. Global change of gene expression and cell physiology in YidC-depleted *Escherichia coli*. J Bacteriol **192:**2193–2209. http://dx.doi.org/10.1128/JB.00484-09.
21. **Typas A, Banzhaf M, Gross CA, Vollmer W.** 2011. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Microbiol **10:**123–136. http://dx.doi.org/10.1038/nrmicro2677.
22. **Yang DC, Peters NT, Parzych KR, Uehara T, Markovski M, Bernhardt TG.** 2011. An ATP-binding cassette transporter-like complex governs cell-wall hydrolysis at the bacterial cytokinetic ring. Proc Natl Acad Sci U S A **108:**E1052–E1060. http://dx.doi.org/10.1073/pnas.1107780108.
23. **Gray AN, Li Z, Henderson-Frost J, Goldberg MB.** 2014. Biogenesis of YidC cytoplasmic membrane substrates is required for positioning of autotransporter IcsA at future poles. J Bacteriol **196:**624–632. http://dx.doi.org/10.1128/JB.00840-13.
24. **van der Laan M, Nouwen N, Driessen AJ.** 2004. SecYEG proteoliposomes catalyze the Δψ-dependent membrane insertion of FtsQ. J Biol Chem **279:**1659–1664. http://dx.doi.org/10.1074/jbc.M306527200.
25. **van der Laan M, Houben EN, Nouwen N, Luirink J, Driessen AJ.** 2001. Reconstitution of Sec-dependent membrane protein insertion: nascent FtsQ interacts with YidC in a SecYEG-dependent manner. EMBO Rep **2:**519–523. http://dx.doi.org/10.1093/embo-reports/kve106.
26. **Urbanus ML, Scotti PA, Froderberg L, Saaf A, de Gier JW, Brunner J, Samuelson JC, Dalbey RE, Oudega B, Luirink J.** 2001. Sec-dependent membrane protein insertion: sequential interaction of nascent FtsQ with SecY and YidC. EMBO Rep **2:**524–529. http://dx.doi.org/10.1093/embo-reports/kve108.
27. **Spratt BG.** 1975. Distinct penicillin binding proteins involved in the division, elongation, and shape of *Escherichia coli* K-12. Proc Natl Acad Sci U S A **72:**2999–3003. http://dx.doi.org/10.1073/pnas.72.8.2999.
28. **Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P.** 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS

Microbiol Rev **32:**234–258. http://dx.doi.org/10.1111/j.1574-6976.2008.00105.x.
29. **Bertsche U, Breukink E, Kast T, Vollmer W.** 2005. *In vitro* murein peptidoglycan synthesis by dimers of the bifunctional transglycosylase-transpeptidase PBP1B from *Escherichia coli*. J Biol Chem **280:**38096–38101. http://dx.doi.org/10.1074/jbc.M508646200.
30. **Bertsche U, Kast T, Wolf B, Fraipont C, Aarsman ME, Kannenberg K, Von Rechenberg M, Nguyen-Distèche M, Den Blaauwen T, Höltje J.** 2006. Interaction between two murein (peptidoglycan) synthases, PBP3 and PBP1B, in *Escherichia coli*. Mol Microbiol **61:**675–690. http://dx.doi.org/10.1111/j.1365-2958.2006.05280.x.
31. **Sauvage E, Derouaux A, Fraipont C, Joris M, Herman R, Rocaboy M, Schloesser M, Dumas J, Kerff F, Nguyen-Distèche M.** 2014. Crystal structure of penicillin-binding protein 3 (PBP3) from *Escherichia coli*. PLoS One **9:**e98042. http://dx.doi.org/10.1371/journal.pone.0098042.
32. **Di Lallo G, Fagioli M, Barionovi D, Ghelardini P, Paolozzi L.** 2003. Use of a two-hybrid assay to study the assembly of a complex multicomponent protein machinery: bacterial septosome differentiation. Microbiology **149:**3353–3359. http://dx.doi.org/10.1099/mic.0.26580-0.
33. **Ploeg R, Verheul J, Vischer NO, Alexeeva S, Hoogendoorn E, Postma M, Banzhaf M, Vollmer W, Blaauwen T.** 2013. Colocalization and interaction between elongasome and divisome during a preparative cell division phase in *Escherichia coli*. Mol Microbiol **87:**1074–1087. http://dx.doi.org/10.1111/mmi.12150.
34. **Zhao G, Meier TI, Kahl SD, Gee KR, Blaszczak LC.** 1999. Bocillin-FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. Antimicrob Agents Chemother **43:**1124–1128.
35. **Hatzixanthis K, Palmer T, Sargent F.** 2003. A subset of bacterial inner membrane proteins integrated by the twin-arginine translocase. Mol Microbiol **49:**1377–1390. http://dx.doi.org/10.1046/j.1365-2958.2003.03642.x.
36. **Chen JC, Minev M, Beckwith J.** 2002. Analysis of *ftsQ* mutant alleles in *Escherichia coli*: complementation, septal localization, and recruitment of downstream cell division proteins. J Bacteriol **184:**695–705. http://dx.doi.org/10.1128/JB.184.3.695-705.2002.
37. **Chen JC, Weiss DS, Ghigo JM, Beckwith J.** 1999. Septal localization of FtsQ, an essential cell division protein in *Escherichia coli*. J Bacteriol **181:**521–530.
38. **van der Does C, de Keyzer J, van der Laan M, Driessen AJ.** 2003. Reconstitution of purified bacterial preprotein translocase in liposomes. Methods Enzymol **372:**86–98. http://dx.doi.org/10.1016/S0076-6879(03)72005-9.
39. **Saller MJ, Fusetti F, Driessen AJ.** 2009. *Bacillus subtilis* SpoIIIJ and YqjG function in membrane protein biogenesis. J Bacteriol **191:**6749–6757. http://dx.doi.org/10.1128/JB.00853-09.
40. **Gesteland RF.** 1966. Isolation and characterization of ribonuclease I mutants of *Escherichia coli*. J Mol Biol **16:**67–84. http://dx.doi.org/10.1016/S0022-2836(66)80263-2.
41. **Keller R, de Keyzer J, Driessen AJ, Palmer T.** 2012. Co-operation between different targeting pathways during integration of a membrane protein. J Cell Biol **199:**303–315. http://dx.doi.org/10.1083/jcb.201204149.
42. **Reddy M.** 2007. Role of FtsEX in cell division of *Escherichia coli*: viability of *ftsEX* mutants is dependent on functional SufI or high osmotic strength. J Bacteriol **189:**98–108. http://dx.doi.org/10.1128/JB.01347-06.
43. **Price CE, Driessen AJ.** 2008. YidC is involved in the biogenesis of anaerobic respiratory complexes in the inner membrane of *Escherichia coli*. J Biol Chem **283:**26921–26927. http://dx.doi.org/10.1074/jbc.M804490200.
44. **Zijderveld CA, Aarsman ME, den Blaauwen T, Nanninga N.** 1991. Penicillin-binding protein 1B of *Escherichia coli* exists in dimeric forms. J Bacteriol **173:**5740–5746.
45. **Weiss DS, Chen JC, Ghigo JM, Boyd D, Beckwith J.** 1999. Localization of FtsI (PBP3) to the septal ring requires its membrane anchor, the Z ring, FtsA, FtsQ, and FtsL. J Bacteriol **181:**508–520.
46. **D'Ulisse V, Fagioli M, Ghelardini P, Paolozzi L.** 2007. Three functional subdomains of the *Escherichia coli* FtsQ protein are involved in its interaction with the other division proteins. Microbiology **153:**124–138. http://dx.doi.org/10.1099/mic.0.2006/000265-0.
47. **Hyyryläinen H, Marciniak BC, Dahncke K, Pietiäinen M, Courtin P, Vitikainen M, Seppala R, Otto A, Becher D, Chapot-Chartier M.** 2010. Penicillin-binding protein folding is dependent on the PrsA peptidyl-prolyl *cis-trans* isomerase in *Bacillus subtilis*. Mol Microbiol **77:**108–127. http://dx.doi.org/10.1111/j.1365-2958.2010.07188.x.



Supplemental FIG. 1. Immunoblot analysis of PBP3 and PBP1B. IMVs with and without YidC or FtsQ were loaded in duplicate on a gel and blotted onto a single membrane. The membrane was divided in the middle (arrows) and the left part was developed with anti-PBP3 and the right part with anti-PBP1B. Both monomer and higher Mw forms are detectable for both proteins. The higher Mw bands run at different heights and might correspond to dimers or complexes with other proteins, but not to a PBP3/PBP1B complex.